IMMUNOAFFINITY EXTRACTION

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Introduction

The mechanisms of separation in liquid chromatography are often classified as adsorption, partition, ion exchange and size exclusion. A further category could be included – affinity separations. Affinity chromatography uses very specific interactions between the compound of interest and a ligand bound to a chromatographic support to obtain separations. An early example of affinity separations was the use of an enzyme and its substrate. One particular type of affinity separation is immunoaffinity chromatography. In this case antibody–antigen interactions are used to obtain the separation. Either the antibody or the antigen can be bound to a support (immobilized). The current use of immunoaffinity extraction usually has the antibody immobilized (**Figure 1**). Immunoaffinity chromatography has often been used in the preparative mode where molecules of biological interest, which are difficult to recover by other methods, have been purified. Examples include enzymes, hormones, vaccines, interferons and antibodies.

Many modern analytical methods involve at least two distinct stages: preparation of a sample in a relatively clean form followed by instrumental analysis. This is particularly the case for the measurement of low concentrations of organic compounds in complex biological matrices such as blood, plasma, serum, urine, tissues and environmental matrices such as water, air, soil, foods, etc. One reason for the current interest in immunoaffinity extraction is its potential use as a highly specific variant of traditional solid-phase extraction in such analyses. In an ideal immunoextraction the sample is added to the column and only the target analyte is retained on the column. A wash step is then incorporated and potentially interfering material in the sample is washed from the column and discarded. The solvent is then changed and the elution solvent removes the target analyte from the column. The clean eluent is then analysed, usually by a modern instrumental method such as high performance liquid chromatography (HPLC) or gas chromatography (GC). This principle is shown in **Figure 2**. Immunoaffinity extraction is thus an attempt to combine the specificity of antibody-based methods with the separation and selective detection that can be obtained from instrumental chromatographic methods.

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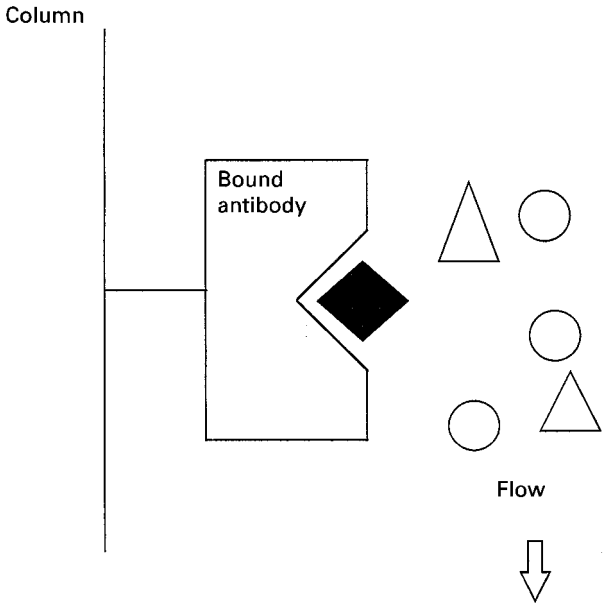

Figure 1 Principle of affinity extraction. Only analyte binds to the antibody. ♦, analyte; △, ○, unrelated compounds.

Solid-Phase Extraction

Solid-phase extraction is one of the most common forms of sample preparation in current use. In its usual format, it involves introducing a liquid sample to an extraction cartridge in a small syringe-shaped container. The cartridge contains a solid phase capable of extracting the analytes of interest and retaining them on the solid phase. The analyte is thus removed from a 'dirty' matrix. It is then eluted from the solid phase and injected into a GC or an HPLC. Such a procedure produces a cleaner sample and therefore less likelihood of peaks co-eluting with the analyte. The liquids are normally drawn through the cartridge under vacuum using a purpose-designed vacuum box, or using positive pressure at the head of the column. Solid-phase extraction is a simple form of liquid chromatography. A range of phases is commercially available, such as silica, C_{18}, C_8, C_5, C_2, phenyl, diol, amino bonded silica, ion exchange phases and polymer phases. Conventional solid-phase extraction

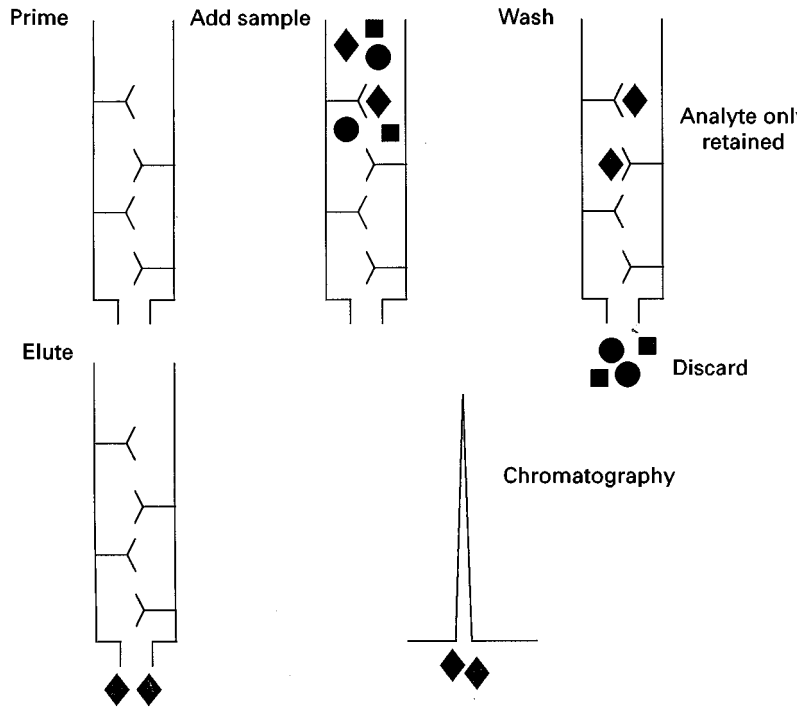

Figure 2 Idealized immunoextraction. Only analyte (♦) is retained through the wash step. This is then eluted and subsequently injected onto HPLC, GC, etc.

is easy to automate both online and offline. Commercially available phases have been used to analyse thousands of different compounds, but generally they are nonselective about which analytes they extract. A range of tailor-made phases have been developed, designed to extract only one or a few closely related analytes. Immunoaffinity extraction is an example of an attempt to develop highly specific solid-phase extraction procedures.

Antibodies

The key reagent for immunoextraction is the antibody which is immobilized on to a support. Antibodies are large biological molecules present in the serum of animals. They are produced by the immune system in response to foreign compounds, the so-called antibody–antigen response. Antibodies belong to a group of proteins called the immunoglobulins and have a relative molecular mass of about 150 000–900 000. Antibodies are normally only produced in response to compounds with a molecular mass of 1000 or above. As many of the compounds of interest in analytical chemistry are much smaller than this, they are chemically bonded to a carrier protein in order to elicit the immune response. For the antibody to be useful it must respond to the analyte, not to the analyte–protein complex alone. In cases where the analyte does not contain a functional group suitable for bonding to a carrier protein, a structural analogue to the analyte is sometimes evaluated. As serum containing the antibodies is collected, it is referred to as antiserum. It will contain a number of different antibodies and is known as a polyclonal antibody.

In practice these antibodies will bind compounds bearing a close structural relationship to the compound of interest. This is known as cross-reactivity, and can be useful in immunoextraction as a group of compounds, such as phenylurea pesticides, can be extracted and then subsequently separated by HPLC. The forces involved in the antibody–antigen interaction are a mixture of ionic attraction, hydrogen bonding, hydrophobic attractions and van der Waals forces. Although individually they are relatively weak forces, in combination a relatively strong attraction is achieved. As a chemical reagent, antibodies are not very stable. They are easily denatured by extremes of pH and by organic solvents. They are much more stable under physiological conditions (i.e. close to pH 7 and in saline at about 1%). The main attraction of biological antibodies in analytical chemistry is their specificity, which arises due to biological recognition at the molecular level.

Table 1 Support materials used for antibody immobilization

Dextran (α-1,6-linked glucose)
Agarose (poly galactose and anhydro-galactose)
Cellulose (1,6-linked glucose chains)
Polyacrylimide
Alumina
Silica
Controlled pore glass

Immobilization of Antibody

Immunoextraction columns require the bonding of an antibody on to a suitable support while retaining the maximum amount of antibody activity. Some of the support materials used are shown in **Table 1** (for their particular characteristics, see Godfrey in Further Reading). Ideally, supports should show good flow characteristics, good chemical and mechanical stability, low nonspecific adsorption, low cost and suitable functional groups for bonding the antibody. As with conventional HPLC and solid-phase extraction, silica-based sorbents are the most popular for immunoextraction.

The methods used to couple antibodies to sorbents usually involve reaction with the carboxyl or amino groups on the antibodies. A range of different reagents is used to activate the sorbent on to which the antibody is bound. These include cyanogen bromide, carbonyl diimidazole, 1,4-butanediol diglycidoxy ether, divinyl sulfone, tresyl chloride and glutaraldehyde. Sorbents need to be thoroughly washed after bonding to remove residual reagents.

Optimization of Immunoextraction

Once an immunosorbent has been prepared, a known amount (by weight or volume) is added to a plastic or glass column with a retaining frit. As immunosorbents are relatively expensive to prepare (compared with commercially available solid-phase extraction columns), the minimum amount needed for a particular assay is used. Conditions are also chosen to allow the columns to be used many times. This means that gentle extraction conditions are favoured, otherwise the antibody will be denatured. However, when using immunoaffinity extraction as a clean-up method for chromatography, it is also desirable to elute the analyte in as small a volume as possible so that further preconcentration is unnecessary. A further consideration is the nature of the desorbing eluent with respect to the possibility of direct injection into an HPLC or GC. In principle there are many different variables requiring optimization for a successful immunoextraction (**Table 2**).

As the actual column preparation follows established protocols, much of the effort of developing a successful extraction protocol concentrates on the solvents used for conditioning and washing the column and on the solvent used for elution of anlyte. Columns typically contain between 50 and 500 μL of antiserum. As antibodies orginate from animal serum, physiological conditions are favourable for column washing.

A typical protocol would prime the column with phosphate-buffered saline at neutral pH. The sample would be loaded at a pH adjusted to fall in the range pH 5–9. Large sample volumes can be added and this allows concentration of the analyte on the column. The capacity of immunocolumns is usually dictated by the mass of analyte rather than the volume of sample. Biological samples such as plasma or serum often cause column blockage unless proteins are precipitated before the sample is added to the column. Flow rates up to about 5 mL min^{-1} are acceptable; otherwise, with higher flow rates the antibody–antigen interaction does not have sufficient time to ensure binding.

Once the analyte is bound on to the column it can be washed with phosphate-buffered saline at neutral pH. In order to elute the analyte in as small a volume as possible, without damaging the antibody, elution solvents are typically composed of phosphate-buffered saline at a low pH (down to pH 2) with the addition of a water-miscible solvent such as methanol or ethanol at a concentration of up to about 50%. At higher pH or lower concentration of organic modifier, the analyte elutes in a larger volume of elution solvent, which necessitates further concentration. This type of desorption is known as nonselective desorption. An alternative approach is to try selective desorption by adding a compound very similar in structure to the analyte in the elution solvent. This

Table 2 Parameters for optimization of immunoaffinity extraction

Type of support
Activation of support
Particle size of support
Pore size of support
Amount of antibody to immobilize
Immobilization chemistry
Quality and purity of antibody
Column dimensions
Column priming
Volume of sample to load
pH of sample
Wash solvent composition including pH
Elution solvent composition including pH
Flow rate
Regeneration conditions

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approach has generally necessitated larger desorption volumes to obtain quantitative recovery than non-selective desorption and hence is not common.

One of the advantages of immunoaffinity extraction is that these procedures are usually carried out under aqueous conditions. As reversed-phase HPLC is often the favoured technique for subsequent analysis, the introduction of analyte dissolved in an aqueous medium is compatible with the mobile phase. Most applications of the technique involve extraction of drugs, endogenous compounds and pesticides. Examples of compounds for which immunoaffinity extraction has been reported are shown in **Table 3**.

One of the most successful applications of immunoaffinity has been the extraction of pesticides from water. Immunoextraction columns have been shown to be capable of preconcentrating up to 1 L of water containing the herbicides chlortoluron and isoproturon, yet still capable of desorption into low volumes (even as low as 1 mL) of elution solvent. Although lower sample volumes (such as 50–100 mL) are more likely to be used in practice, this feature of immunocolumns offers the possibility of large concentration factors and low overall detection limits. The cross-reactivity of antibodies to triazines and phenylureas has been used to immunoextract several compounds which were then subsequently separated and measured by HPLC.

The capacity of the immunocolumns is governed by the mass of analyte that can be retained before the column is overloaded rather than the volume of water passed through. The mass capacity of the column can be assessed by loading 1 mL aliquots of a standard solution of analyte and analysing fractions eluting from the column until the presence of analyte is detected. A simple calculation of the number of additions to the column times the amount added each time gives the mass breakthrough of analyte. An alternative approach involves overloading the column but then washing out excess analyte in solution, leaving bound analyte on the column. This is then eluted with the desorbing solution and the concentration and volume measured. A simple calculation gives the amount of analyte required to saturate the column.

Table 3 Examples of immunoaffinity extraction

Aflatoxins
Albuterol
Atrazine
Carbofuran
Chloramphenicol
Ciprostene
Clenbuterol
Cytokinins
Isoproturon
Morphine
Ochratoxin A
Propranolol
Prostaglandins
Steroids
Thromboxane metabolites
Tenbolone
Zeranol

The major advantage of immunoaffinity columns is the specificity that can be obtained. This is utilized to give cleaner chromatographic traces than using nonselective extraction such as liquid–liquid extraction or solid-phase extraction on silica or non-polar bonded silica.

Other Formats of Immunoaffinity Extraction

Immunoaffinity extraction has been carried out in formats other than solid-phase extraction. This has included high performance immunoaffinity chromatography and online HPLC column switching. In the former, immunosorbents are used as HPLC columns whereas in the latter, samples are extracted on an HPLC immunosorbent, preconcentrated and the flow then switched to a conventional HPLC column for analysis. Both methods attempt to base the separation on antibody–antigen interactions. In the case of column switching, complete automation can be achieved.

Molecular Imprinted Polymers

The major disadvantage with immunoaffinity extraction is the difficulty and expense in obtaining biological antibodies. An alternative approach is the use of molecular imprinted polymers as antibody mimics. These are synthesized in the chemistry laboratory and are consequently easier and less expensive to obtain. The target analyte (template) is mixed with a monomer such as methyl acrylic acid and a cross-linking agent such as ethylene glycol dimethacrylate. They are dissolved in a suitable solvent such as acetonitrile along with an initiator such as 2,2′-azobis-(2-methylpropionitrile) and heated or subjected to UV radiation. The polymer forms around the template within about 16 h. The polymer is ground into fine particles and then washed to remove the analyte template, thereby leaving cavities where the analyte can subsequently be bound. This polymer can then behave as an affinity column, mimicking the biologically derived immunoaffinity solid-phase extraction columns.

Columns derived from molecular imprinted polymers often show secondary interactions arising from the monomer, e.g. with methyl acrylic acid, cation exchange can occur. They show best specificity in the solvent in which they were originally dissolved, hence they are used with organic rather than aqueous solvents. Although easy to obtain and more stable to extremes of pH and organic solvents, they are not as specific as columns utilizing biologically derived antibodies. One problem with molecular imprinted polymers is the difficulty in washing out all traces of the analyte template. Remaining template leaches out when the columns are used for analysis, giving falsely high results. This problem is partially overcome by using a structural analogue to the analyte as the template. Provided the template can be separated from the analyte by HPLC, GC, etc. it will not interfere with the analysis. This approach does require cross-reactivity of the polymer, i.e. it must retain the analyte as well as the template.

The use of molecular imprinted polymers is an emerging field and new synthetic methods may improve the performance of these columns as well as other uses of the polymers. Examples of solid-phase extraction using molecular imprinted polymers include atrazine, pentamidine, propranolol, sameridine and tamoxifen.

Future Developments

Immunoaffinity extraction has been demonstrated as being capable of selectively capturing analytes from complex matrices using antibody–antigen interactions. Techniques for preparing the columns and procedures for optimizing the retention and desorption of analyte are now well established. The availability of antisera to more compounds will expand the use of immunoextraction. As better procedures to produce antibodies or antibody fragments become available, the cost of antisera should come down. Although much of the work to date uses low molecular weight compounds as target analytes, immunoextraction might be even more valuable for the new products emerging from biotechnology which may present different problems with extraction using conventional liquid–liquid or solid-phase extraction methods. Better specificity from synthetic polymer antibody mimics should also see a growth in their utility in immunoaffinity-type extractions. Polymers that show specificity for analyte under aqueous conditions would be an advantage. Selective extraction at present comes at an extra cost in the production of the columns and is not yet available 'off the shelf'. It is likely to prove most useful where simpler procedures cannot be used due to analyte instability or where particularly low detection levels are required. It should also be remembered that immunoaffinity extraction need not only be used with HPLC, GC or capillary electrophoresis.

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